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Award Number: DAMD17-00-1-0570

TITLE: Characterization of the Chicken Ovarian Cancer Model

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REPORT DATE: August 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2002	Annual (15 Jul 01 - 14 Jul 02)

4. TITLE AND SUBTITLE
Characterization of the Chicken Ovarian Cancer Model

5. FUNDING NUMBERS
DAMD17-00-1-0570

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*
We believe that the domestic laying hen has great potential as an animal model for ovarian cancer prevention research. Unlike other ovarian cancer models, which require experimental induction of ovarian tumors, chickens develop ovarian adenocarcinoma spontaneously, with an incidence ranging from 13 to 40 percent between four and six years of life. No investigators have taken advantage of the chicken to study ovarian cancer. Thus, the chicken ovarian cancer model has yet to be validated and developed. As part of a prevention trial in chickens funded by the Department of Defense, we have accumulated 1400 chicken reproductive tracts including 140 with adenocarcinomas, and gathered valuable data regarding the natural history of these tumors. This provides the remarkable opportunity to critically evaluate the chicken ovarian cancer animal model and determine its relevance to human ovarian cancer research. The aim of the current proposal is to increase our understanding of the molecular and histologic features of chicken ovarian cancers. In addition, we will develop a histologic classification for chicken ovarian cancers, which is a critically important prerequisite to the widespread use of this animal model for ovarian cancer research.

14. SUBJECT TERMS	15. NUMBER OF PAGES
ovarian cancer, animal model, chemoprevention	11
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Introduction

Epithelial ovarian cancer is a highly lethal malignancy. It is the fifth leading cause of cancer deaths among women in the United States and causes 140,000 deaths annually in women worldwide. Despite intensive research efforts over the past decade directed towards improved detection and treatment, the long-term survival of women with ovarian cancer has only improved modestly. Progress in the fight against ovarian cancer has been hampered by a number of factors, including late diagnosis, molecular heterogeneity of tumors, absence of highly curative chemotherapy, and lack of a valid animal model.

We believe that development of effective chemopreventive agents for ovarian cancer represents our best hope for decreasing ovarian cancer mortality in the future. Based on our studies in primates and in the laboratory, we are convinced that the well-known protective effect of oral contraceptives against ovarian cancer is due, in large part, to the molecular biologic effects of progestins on the ovary. We have found that progestins activate the apoptosis pathway in the ovarian epithelium, making it more likely that cells that have incurred genetic damage will be eliminated, rather than develop into cancer. A number of other apoptosis-inducing agents also hold promise for preventing ovarian cancer, including retinoids. Ultimately, it is our goal to develop a preventive strategy using the best chemopreventive agents, either alone or in combination, in order to achieve maximum protection against ovarian cancer.

At this time, the lack of a valid ovarian cancer animal model is a major obstacle to ovarian cancer prevention research. In order to develop pharmacologic preventive strategies for ovarian cancer in a timely fashion, animal models that closely mimic human ovarian cancer are desperately needed. Human prevention trials are costly requiring large numbers of subjects and many years to complete. Development of an animal model for ovarian cancer prevention research would represent a significant breakthrough and lead to expedited evaluation of numerous candidate agents. Ideally, this would lead to rapid identification of a select number of agents, which have the greatest potential for ovarian cancer prevention and that can then be evaluated in human prevention trials.

We believe that the domestic laying chicken has great potential as an animal model for studying chemoprevention of ovarian cancer. Unlike other animal models for ovarian cancer, which generally require the experimental induction of ovarian tumors, the chicken develops ovarian cancer spontaneously. The domestic hen is the only animal with a high incidence of spontaneous ovarian adenocarcinoma, ranging from 13 to 40 percent between four and six years of life. No investigators have taken advantage of the chicken to study ovarian cancer. Thus, the chicken ovarian cancer model has yet to be validated and developed. As part of a chemoprevention grant awarded to us by the Department of Defense in 1998, we have performed a two-year chemoprevention trial in the chicken designed to test the hypothesis that progestins confer chemopreventive effects against ovarian cancer. We are now conducting a second prevention trial in the chicken, funded by the NIH, evaluating progestin and the retinoid 4-HPR as candidate preventives.

We have accumulated 140 chicken reproductive tract cancers, including 98 ovarian tumors, from our first prevention trial, and gathered valuable data regarding the

natural history of these tumors. In addition, we are gathering both tumor and natural history data from our second prevention trial that is underway. Data and tissues that we are gathering provide us with the remarkable opportunity of being able to critically evaluate the chicken ovarian cancer animal model and determine its relevance to human ovarian cancer research. The aim of the current proposal is to increase our understanding of the molecular and histologic features of chicken ovarian cancers. In addition, we will develop a histologic classification for chicken ovarian cancers, which is a critically important prerequisite to the widespread use of this animal model for ovarian cancer research. For this proposal, we plan to characterize and develop the chicken ovarian cancer model by (1) analyzing the molecular and genetic features of chicken ovarian cancers, including alterations in the p53 tumor suppressor gene and the Her-2/neu and Ras oncogenes, (2) classifying the morphologic and histologic features of chicken ovarian cancers, leading to the development of a histologic classification for chicken ovarian adenocarcinomas, and (3) comparing the molecular and histologic features of ovarian cancers that develop in chickens receiving synthetic progestins compared to untreated controls. We hope to gather evidence that chicken ovarian cancers have genetic alterations and morphologic features similar to those identified in human ovarian carcinomas, thereby validating the chicken ovarian cancer model.

BODY:

Aim 1: To determine whether the genetic alterations that characterize ovarian cancers in women are also a feature of ovarian cancers in the domestic fowl.

We have made significant progress in accomplishing the tasks within this aim. In last year's update, we reported that we had performed extraction of DNA and RNA from over 100 frozen tumor samples derived from avian reproductive tract (ovary and oviduct) cancers. Primers were designed for p53, H-Ras, and K-ras. Reverse transcriptase PCR has been used to amplify the p53 gene. The entire coding region has been screened for alterations using single stranded conformational analysis and direct sequencing of variant bands has been performed. Clearly deleterious p53 mutations have been found in approximately 20% of chicken reproductive tract cancers. The majority of these (93%) are insertions deletions that predict truncated protein products. There was one missense mutation that results in an amino acid substitution. All the mutations were located between amino acids 101 and 336, which correspond to the DNA binding domains. Four unique silent single nucleotide polymorphisms were detected in 9 of the cancers. An identical 22-base pair insertion polymorphism in the 3-prime untranslated region of the gene was identified in 14 samples. Over the next year, we plan to evaluate the association between p53 mutation and reproductive tract cancer stage as well as the influence of hormonal treatment with progestin on the incidence of p53 mutations. We also plan to compare the p53 mutation profile in both the oviduct and ovary in chickens that have tumors at both sites to further determine whether the tumors are distinct versus arising from a common precursor. The finding of frequent mutations in p53 in chicken reproductive tract cancers is similar to what has been described in reproductive tract cancers in women, which is evidence in support of the chicken as a valid animal model

for ovarian cancer research. Chicken H-ras primers were designed for region of the gene containing codons 12 and 13 from the known sequence (Westway et al., Acc. No. X03578). Eighty-nine ovarian cancers have been screened. Only one tumor had a polymorphic base (GGT→GGC) in codon 12 while none were found in codon 13. For chicken K-Ras, primers were developed based on the reported sequence *for M. gallopavo* (turkey). The resulting 175-bp amplicon shares 90% and 99% homology to human and turkey K-Ras, respectively. There were no polymorphic bases in either codon 12 or 13 for 89 sequenced samples. There was, incidentally, a silent polymorphic base in codon 39 (TC<u>G</u>→TC<u>C</u>) with a rare allele frequency of 0.36. The frequency of H- and K-Ras mutations in invasive chicken ovarian cancers is, thus, not unlike that found in humans.

The optimal staining methods for Her-2-neu are currently being worked up in preparation for staining for Her 2-neu.

As the studies outlined in Aim #2 are now maturing, the pattern of gene expression and mutation will be compared among tumors from birds subjected to different preventive treatments to see if treatment characteristics influence the genotype of reproductive tract cancers in the chicken.

Aim 2: To classify the morphologic and histologic features of chicken ovarian cancers, leading to the development of a histologic classification of chicken ovarian adenocarcinomas.

Significant progress has been made in the histologic evaluation and classification of reproductive tract tumors accrued from the chemoprevention study funded by the Department of Defense (DAMD 17-98-1-8686) under our prior Duke Program Project grant. A registry of 154 adenocarcinomas obtained from 1405 4-yr-old laying hens has been established and is being used to develop the proposed classification scheme. Among the tumors in the registry are ones that occurred only in the ovary, only in the oviduct, in both the ovary and oviduct, in multiple visceral organs, or in visceral organs without involvement of either the ovary or oviduct (see Table 1). The reproductive tract (ovary or oviduct) was involved in over 97% of the hens. Nearly equal numbers of birds had tumors in the ovary or oviduct. Approximately twice as many hens had tumors in both the ovary and oviduct than tumors only in the ovary or oviduct. It is clear that adenocarcinomas in the ovary and oviduct are closely related to one another. The nature of this relationship and significance to the chicken model of ovarian cancer remain to be resolved. In the chemoprevention study from which the registry cases were obtained (see Figure 1), cancers occurring in either the ovary or oviduct were similarly affected by the interventions suggesting that they have a common pathogenesis. It appears likely that not only are ovarian adenocarcinomas an avian homologue of ovarian cancers in women, but the oviductal adenocarcinomas may represent the avian equivalent of fallopian tube or uterine cancers.

The identity of the cell or cells of origin of the tumors remains elusive. Previously, it has been considered that if a tumor exists in the oviduct, no matter how small or well differentiated, any ovarian tumor in that same bird is secondary to the oviductal adenocarcinoma. However, evidence we are gathering based on adenocarcinomas in the

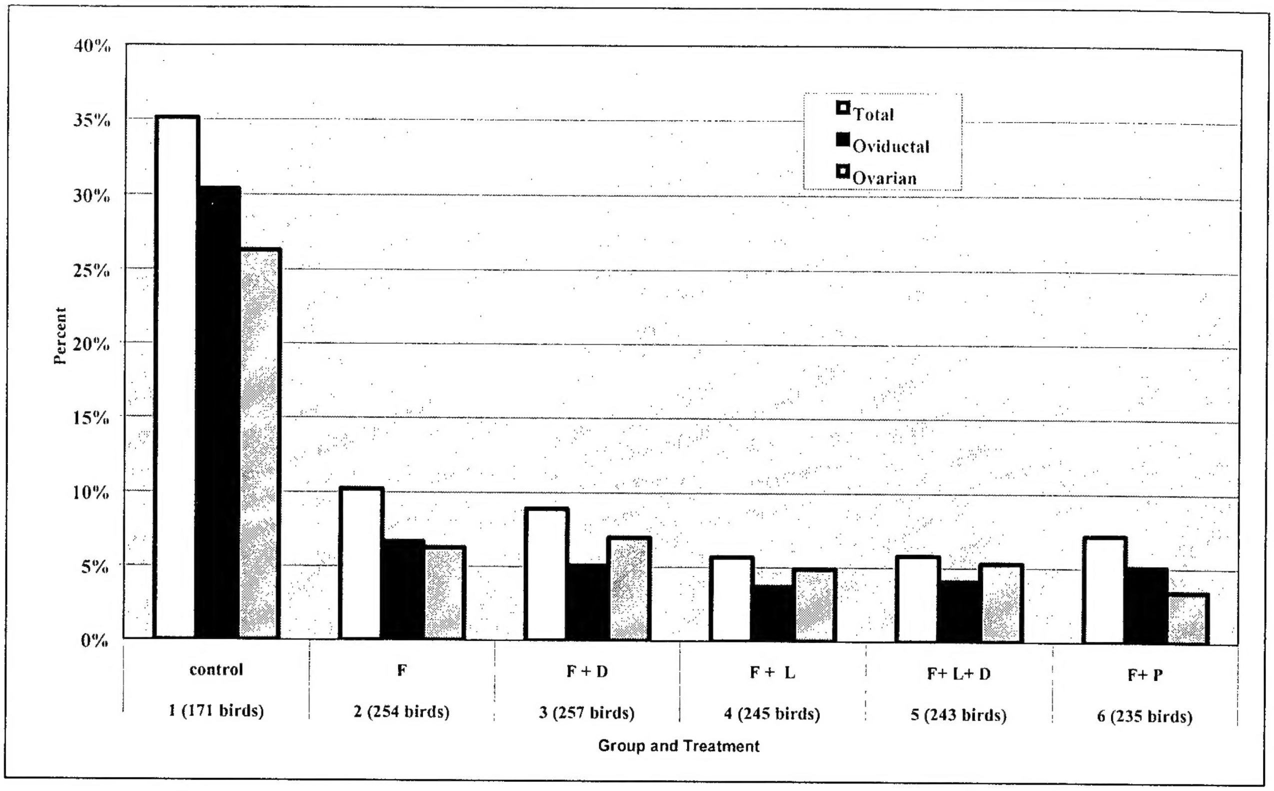
ovary with characteristics of oviductal-type cells, from birds that have no tumor in the oviduct, indicate that either the cells that develop into adenocarcinomas in the ovary have the capacity to differentiate into an oviductal phenotype or rests of oviductal cells remain in the ovary that can become transformed. We believe the former is most likely, as we have found no evidence for ectopic oviductal glandular epithelium in over 700 normal chicken ovaries. Further, this hypothesis is inconsistent with cases in which most of the ovary is cancerous whereas only a small tumor is present in the oviduct. Lastly, our histopathologic evaluations indicate that cancerous cells with an oviductal character (ovalbumin granules in the cytoplasm) are likely responding to the same hormonal signals as normal oviductal glandular epithelium; tumor cells are more likely to have cytoplasmic granules if the oviduct is functional and less likely to have them if the tract is involuted. We believe the ovarian cancers of the chicken, like those of women, develop cytologic features in common with cells elsewhere in the reproductive tract and that in the chicken, this is the oviductal glandular epithelium. Furthermore it is most likely that the chicken commonly has synchronous tumors that arise multicentrically in the ovary, oviduct, and/or other visceral organs. Synchronous tumors have been described in women with adenocarcinomas of both the ovary and uterus.

A critical aspect of this work to validate the chicken model of ovarian cancer in women is to have a clear definition of what constitutes an ovarian adenocarcinoma. Substantial progress has been made on this important point. We have developed a 3-tiered grading system for identifying the likelihood that an ovarian cancer in a chicken is primary or secondary based on the histopathologic evaluation of all ovarian cancers in the registry. Grade 1 adenocarcinomas of the ovary are multifocal, nodular, involve only the cortex, and affect less than 25% of the ovary. They are interpreted to be secondary and not primary tumors, as they do not differ in appearance from serosal implants that commonly accompany adenocarcinomas in chickens. Grade 2 adenocarcinomas are multifocal to diffuse, may involve either the medulla or cortex or both, and affect between 25 – 75% of the ovary. These are interpreted to be probable primary ovarian adenocarcinomas. Grade 3 tumors are similar to grade 2 tumors except they tend to be diffuse, always involve the medulla, and affect greater than 75% of the ovary. We have a high degree of confidence defining grade 1 tumors as secondary and grade 3 tumors as primary, but are less confident about the interpretation of grade 2 tumors. These will be studied in more detail to see if the grading system can be further refined. Currently we are considering both grade 2 and grade 3 tumors to be adenocarcinomas of probable ovarian origin.

Remaining, is completion of the detailed morphologic evaluation of all tumors stained by conventional methods, which is in progress. Additionally 5 representative adenocarcinomas from birds with only ovarian or oviductal cancers, cancers in both the ovary and oviduct, and cancers of uncertain origin will be selected to form a group of 20 cases for detailed histochemical and immunohistologic study. We believe this will result in further refinement of the classification of reproductive tract adenocarcinomas in chickens, identify the comparative features of these tumors with those in women, and establish a basis for accurately identifying and interpreting lesions in future studies.

Table 1. Adenocarcinomas in 1405 4-yr-old laying hens.

All Adenocarcinomas		154
	%	*100*
Ovarian Adenocarcinoma		112
	%	*72.7*
Grade 1 Ovarian Adenocarcinoma		33
	%	*21.4*
Grade 2 Ovarian Adenocarcinoma		28
	%	*18.2*
Grade 3 Ovarian Adenocarcinoma		51
	%	*33.1*
Grade 2 or 3 Ovarian Adenocarcinoma		79
	%	*51.3*
Oviductal Adenocarcinoma		113
	%	*73.4*
Reproductive Tract Adenocarcinoma		150
	%	*97.4*
Ovarian + Oviductal Adenocarcinoma		75
	%	*48.7*
Only Ovarian Adenocarcinoma		37
	%	*24.0*
Only Oviductal Adenocarcinoma		38
	%	*24.6*



F= Feed Restricted, D= Vitamin D, L= Levonorgestrel, P= Provera (Medroxyprogesterone acetate)

Figure 1. Total adenocarcinomas and adenocarcinomas in the oviduct and/or ovary of 4-yr-old laying hens. Almost half of the birds had tumors in both the ovary and oviduct.

Key research accomplishments

- Basic techniques established
- Tumor collection that will form the basis for studies established
- Initial characterization of reproductive tract tumors identifying morphologic affinities between well-differentiated tumor cells and normal cells of oviduct glandular epithelium
- Established the likely relationship between oviduct and ovarian cancers and proposed the theory of a multicentric origin of tumors
- Developed criteria for identifying different tumor types
- Validation of methods underway

Reportable outcomes

1) The data regarding the genetic and histomorphologic features of chicken reproductive tract cancers is now maturing. Over the next year, we expect to continue the work outlined in the grant, including data analysis, and to submit several manuscripts for publication.
2) Funding was applied for and granted by the NCI Prevention branch for a prevention trial in the chicken, evaluating the candidate preventives 4-HPR (a retinoids derivative) and levonorgestrel (a progestin). The trial is scheduled to last two years; the primary outcome measure will be the incidence of reproductive tract tumors. In addition, tumors collected during the trial will be analyzed for p53, Ras, and Her-2 neu, similar to what is planned for the current grant funded by the DOD. The work funded by the NCI will thus provide additional chicken reproductive tract tumor specimens for molecular and analysis, and also allow us to examine whether tumors that arise in birds on different types of hormonal treatment have different molecular phenotypes.
3) We have applied for funding from the Department of Defense under the Duke Program Project Renewal (7/2001) for studies that will include an avian prevention trial in the chicken, evaluating the preventive efficacy of various progestin dosages and schedules, with or without the addition of Vitamin D, on the outcome measure of reproductive tract tumors in the chicken. This funding has been granted.
4) An application was submitted to the RAPID program within the Prevention Branch at the NCI for a broad research plan for development of ovarian cancer preventive agents. The plan includes avian prevention trials in the chicken. The grant was not awarded, but the score was competitive. We plan to resubmit a proposal.
5) Under collaboration with Dr. Bill Cliby at the Mayo clinic, a study has been initiated to examine whether genomic alterations that are common in human ovarian cancers are also common in the chicken. The study complements the work that is being performed under this DOD grant, with the hope that the finding of genomic alterations in chicken ovarian cancers that parallel those in human ovarian cancers would further support and validate the chicken animal model. The collaborative project with Dr. Cliby has been funded with seed money from a grant from the Gynecologic Cancer Foundation.

Conclusions

The availability of a valid ovarian cancer animal model, especially one in which cancers develop spontaneously at a high rate, would represent a critically important breakthrough for ovarian cancer prevention research. An animal which develops spontaneous ovarian cancer would be ideal for ovarian cancer prevention studies, and provide the means through which a large variety of preventive agents can be quickly evaluated, thereby expediting the development of promising chemopreventive agents that could subsequently be tested in human prevention trials. The demonstration that ovarian cancers in the fowl are similar to those seen in women would be a critical step towards

establishing the validity of the chicken model for testing chemopreventive agents. Whereas chemoprevention trials for ovarian cancer in women are difficult because of the relatively low annual incidence of the disease, chickens have a high incidence over a relatively short life span. Demonstration of efficacy of an ovarian cancer chemopreventive strategy in chickens would provide a credible rationale and enthusiasm for testing a similar strategy in women.

Our efforts in developing the chicken ovarian cancer model will help overcome a major obstacle in ovarian cancer prevention research, and provide a means for the rapid development of effective preventives for ovarian cancer.

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